

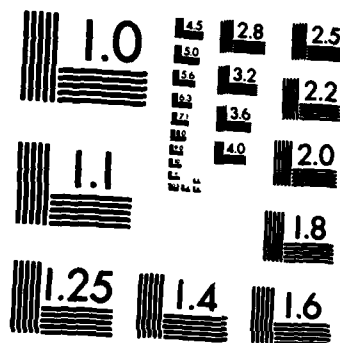
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<p>This report describes the work performed under USAFOSR project #84-0122 which investigated the reactivity of the sympathetic nervous system. During the tenure of this project, the isolated perfused rat adrenal gland preparation was developed as a tool for the examining altered bioreactivity at the molecular level. It was discovered that prior treatment of the animal would altered the response of the adrenal gland to a fixed stimulation protocol. In addition, techniques for the rapid isolation of tyrosine hydroxylase, the rate limiting enzyme in the synthesis of catecholamines was developed. It was established that four distinct protein kinases would phosphorylate this enzyme in at least four distinct sites. Techniques for examining the phosphorylation of this enzyme in tissue that have altered bioreactivity are currently being developed.</p>			
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FINAL TECHNICAL REPORT
U. S. AIR FORCE OFFICE OF SCIENTIFIC RESEARCH PROJECT #84-0122

**BIOCHEMICAL REGULATION OF THE RESPONSE
OF THE SYMPATHETIC NERVOUS SYSTEM**

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1. SUMMARY:

This project has examined molecular mechanisms regulating the reactivity of the adrenal medulla and sympathetic nervous system. The isolated perfused rat adrenal gland preparation was established in this laboratory to evaluate the effects of physiological and environmental manipulation on the responsiveness of the adrenal medulla. Treatments, such as chronic immobilization or cold stress, which result in prolonged adrenal medullary discharge in vivo, result in enhanced adrenal medullary reactivity in vitro as evidenced by the increased secretion of epinephrine from the perfused adrenal gland. The specific biochemical processes underlying this increased reactivity are being investigated using tyrosine hydroxylase, the rate limiting enzyme in the synthesis of the catecholamine neurotransmitters, as a key marker protein. Tyrosine hydroxylase is known to be phosphorylated by four distinct protein kinases in up to four unique sites. The activity of this protein is correlated with tissue levels of catecholamines and its induction is correlated with the enhancement of the bioreactivity of the perfused adrenal gland. Factors that modulate tissue levels of catecholamines and tyrosine hydroxylase are being investigated to determine the mechanism by which they modulate the amount of catecholamines released in response to a fixed dose of acetylcholine.

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2. RESEARCH OBJECTIVES:

The overall goal of the proposed research is to identify the molecular mechanisms that regulate the reactivity of the sympathetic nervous system. Using the isolated perfused rat adrenal medulla, the rate of transmitter biosynthesis, catecholamine release and biochemical changes in key regulatory enzymes have been (will be) examined. Specific objectives of this project include:

- a. Biochemical Characterization of the Effects of Chronic Nerve Activity on the Reactivity of the Adrenal Medulla.
- b. Development of Immunoaffinity Techniques for the Rapid Purification of Tyrosine Hydroxylase.
- c. Phosphopeptide Analysis of Tyrosine Hydroxylase Activated by Nerve Stimulation.

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MATTHEW J. KERPER
Chief, Technical Information Division

d. Examination of the Kinase Responsible for the Activation of Tyrosine Hydroxylase During Neuronal Depolarization.

e. Evaluation of the Biochemical Mechanism of Enhanced Adrenal Responsivity.

3. STATUS OF RESEARCH:

Research efforts from 6/15/84 to 2/28/86 have been focused in five specific areas related to the overall completion of the proposed project. These areas include: a. development of techniques for the perfusion of the isolated rat adrenal gland b. evaluation of the effects of environmental manipulation on reactivity of the adrenal gland c. identification of the phosphorylation sites on tyrosine hydroxylase d. production and characterization of monoclonal antibodies directed against tyrosine hydroxylase and e. development of immunoaffinity purification techniques. These areas of research will be briefly outlined individually below.

a. Development of the Isolated Perfused Rat Adrenal Gland.

The adrenal vein of an anesthetized mature rat is catheterized with P.E. 10 tubing and ligated with 50 silk. The capsule of the adrenal gland is cut on the side opposite the adrenal vein and is perfused with oxygenated Krebs-bicarbonate buffer at a rate of 0.36 ml/min. Following removal of the gland from the animal, the tissue is placed on a plexiglass chamber that is maintained at 38°C and the perfusate collected and assayed for catecholamine content. Prior to quantitation of the acetylcholine dependent catecholamine release, the tissue is perfused for 30 minutes during which time the effects of the surgery and the anesthetic are minimized.

This preparation will release norepinephrine and epinephrine that is directly related to the dose of acetylcholine present in the perfusion medium. Basal secretion rate of release is < 1.0 ng/min. Maximal release of catecholamines at a rate of 50 ng/min was observed during the perfusion of the gland by a bolus injection of 50 ug of acetylcholine.

b. Evaluation of Environmental Manipulation on the Reactivity of the Adrenal Medulla.

Adrenal glands were isolated from rats exposed to a variety of experimental manipulations and examined using the protocol developed above. Exposure of the rats to periods of chronic stress, using immobilization (2 hours/day), exposure to cold (4°C for two hours/day), or chronic hypoglycemia (10 I. U. protamine zinc insulin/day) increased adrenal tyrosine hydroxylase levels, tissue catecholamine levels and an increase in the amount of catecholamine released in following depolarization with acetylcholine.

Treatment of the experimental animals with protamine zinc insulin induces tyrosine hydroxylase and increases plasma levels of catecholamines in response to the hypoglycemia. Adrenal stores of catecholamines are

initially decreased and the amount of catecholamines released from the perfused adrenal gland in response to acetylcholine mediated depolarization is also decreased by a similar magnitude. At seven days following the PZI treatment, the tissue levels of catecholamines is increased, presumably secondary to the induction of TH. The amount of catecholamines released in response to a fixed concentration of acetylcholine appears to be related to the amount of tissue catecholamines present, and the level of tissue catecholamines appears to be related to the degree of induction of tyrosine hydroxylase. Further experiments will be performed to investigate this phenomena.

c. Identification of the Phosphorylation Sites on Tyrosine Hydroxylase.

Highly purified tyrosine hydroxylase can be phosphorylated by cyclic AMP-dependent protein kinase (PKA), calcium and calmodulin-dependent protein kinase (CAM kinase), cyclic GMP-dependent protein kinase (PKG) and calcium and phospholipid-dependent dependent protein kinase (PKC). Tyrosine hydroxylase that has been phosphorylated by each of these kinases using [32 P] ATP and then digested with trypsin (1:10 w/w) for 24 hr at 37°C was analysed by HPLC techniques. Elution of the peptides from a 5 micron C₁₈ reverse phase column equilibrated with 0.1% trifluoroacetic acid with a 0 to 40% acetonitrile gradient demonstrates the presence of two distinct phosphorylation sites. Site A, which is phosphorylated by PKA, PKC, PKG and secondarily by CAM Kinase, is known to be serine 40. Site C, which is preferentially phosphorylated by CAM kinase, is known to be serine 19. Phosphorylation of Site A is associated with an increase in tyrosine hydroxylase activity. The function of the phosphorylation of site C is unknown.

During the investigation of phosphorylation sites on TH, it was discovered that some of the purified preparations of tyrosine hydroxylase were contaminated with trace amounts of an endogenous kinase that would phosphorylate the enzyme at serine 8. Since the sequence around this serine group contains sequences that are not known to be selective for any of the known kinases, it is possible that the kinase that phosphorylates this site is a previously undiscovered kinase. Experiments are being performed to determine if this is a unique kinase and what its physiological function might be.

d. Production of Monoclonal Antibodies Directed Against Tyrosine Hydroxylase.

BA1b/c mice were injected with 20 ug of highly purified tyrosine hydroxylase emulsified in Freund's adjuvant at monthly intervals for a total of six injections. It was found that a serum titer to TH was not obtained until the fourth or fifth injection, suggesting that TH is not very antigenic. A similar observation was made previously during the production of polyclonal antiserum in rabbits. Animals that responded with a sufficient titer were sacrificed, the spleen removed, and the B lymphocytes were flushed from the spleen and fused with mouse myeloma cells by standard techniques. The fused cells were selected for the ability to grow in culture and for the ability to produce IgG. Clones that produced IgG as

demonstrated by ELISA techniques were then assayed for the ability to produce antibodies specifically directed against tyrosine hydroxylase. Six positive clones were obtained that produced antibodies that were able to indirectly precipitate TH. Of these clones, one specific clone, 2D8, appears to be the most promising for the production of monoclonal antibodies for future study, although the other positive clones will be examined in more detail following the complete characterization of the specificity of the antibodies produced by 2D8.

e. Development of Immunoaffinity Purification Techniques for Tyrosine Hydroxylase.

Ascites fluid produced in mice was purified by precipitation to 40% saturation with ammonium sulfate. Following dialysis to remove the salt, the antibodies were coupled to cyanogen bromide activated Sepharose 4B. This matrix was used to attempt immunoaffinity purification of the antigen, tyrosine hydroxylase. Although it appears that this procedure will enable this laboratory to obtain highly purified tyrosine hydroxylase in reasonable amounts, the harsh nature of the conditions necessary for the elution of the the protein precludes the recovery of active protein. An alternate procedure employing affinity chromatography of the antigen antibody complex on Sepharose 4B coupled to S. Aureus protein A is currently being investigated. Preliminary experiments suggest that this will be a more satisfactory method of enzyme purification.

4. WRITTEN PUBLICATIONS:

Campbell, D., Hardie, D. and Vulliet, P. R. Identification of four phosphorylation sites in the N-terminal region of tyrosine hydroxylase. (Submitted to J. Biological. Chem., April, 1986)

Roskoski, R., Vulliet, P. and Glass, D.: Phosphorylation of Tyrosine Hydroxylase by Cyclic GMP-dependent Protein Kinase. (Submitted to J. Neurochem. April, 1986)

Vulliet, P. R., Loskutoff, N. and Kraemer, D. A Technique for embryo transfer in the laboratory rat. Proc. Western. Pharmacol. Soc. (In press, 1986)

Vulliet, P. and Mitchell, J. Altered Reactivity of the Rat Adrenal Medulla Following Periods of Chronic Stress. In: Molecular Mechanisms of Neuronal Responsivity ed by Y. Ehrlich, W. O. Berry and R. H. Lennox, Plenum Press, New York (In Press, 1986).

Kwan, S., Vulliet, P., Denney, R., Shen, R., Westland, K. and Abell, C.: Characterization and Application of Monoclonal Antibodies to Rat Tyrosine Hydroxylase (To be submitted to Molecular Pharmacology, June, 1986).

Shen, R., Hamilton-Byrd, E. Vulliet, P., Kwan, S and Abell, C: An Improved $^{14}\text{CO}_2$ -Trapping Microassay for Tyrosine Hydroxylase Activity in Rat Striatal Synaptosomes. J. Neurosciences Methods, (In Press, 1986).

Vulliet, P. R., Woodgett, J. R., Ferrari, S. and Hardie, D. G.: Characterization of the Sites Phosphorylated on Tyrosine Hydroxylase by the Ca^{2+} and Phospholipid-dependent Protein Kinase, Calmodulin-dependent Multiprotein Kinase and Cyclic AMP dependent Protein Kinase. *FEBS Letters* 182: 335-339, 1985.

Vulliet, P.R.: Direct Activation of Tyrosine Hydroxylase by Calmodulin. *Proc. Western Pharmacol. Soc.* 28: 27-31, 1985.

Vulliet, P.R., Buchl, S.J. and Pelletier, Y.: Inhibition of Tyrosine Hydroxylase following Myocardial Ischemia. *Proc. Western Pharmacol. Soc.* 28: 57-61, 1985.

Hurst, T.W. and Vulliet, P.R.: Uptake of Circulating Epinephrine into Sympathetic Nerve Terminals of Rat Myocardium. *Proc. Western Pharmacol. Soc.* 28: 61-65, 1985.

Mitchell, J. P. and Vulliet, P. R.: Effect of Immobilization on Tyrosine Hydroxylase Activity and Catecholamine Secretion in Rat Adrenal Medulla. *Proc. Western Pharmacol. Soc.* 28: 277-281, 1985.

Vulliet, P.R.: Biochemical Response of the Sympatho-Adrenal Axis. *Proc. Amer. Acad. Vet. Pharmacol. Exp. Ther.* (In Press, 1985).

Vulliet, P.R., Woodgett, J.R. and Cohen, P.: Phosphorylation of Tyrosine Hydroxylase by Calmodulin-dependent Multi-protein Kinase. *J. Biol. Chem.* 259: 13680-13683, 1984.

Vulliet, P.R., Mitchell, J.P. and Hurst, T.W.: Activation of the rat adrenal medulla by direct stimulation of the splanchnic nerve. In: Stress: The Role of Catecholamines and Other Neurotransmitters, ed. by E. Usdin, R. Kvetnansky and J. Axelrod, Gordon and Breach Publishers, New York, pp 481-489, 1985.

5. PROFESSIONAL PERSONNEL:

Philip R. Vulliet, Ph.D., DVM - 50% time
Jo Goy, M.S. - 100% time
Greg Fontenot, M. S.- awarded 12/15/85 - 100% time
Terry Hurst, M. S. awarded on 5/15/86
Jeffrey Mitchell, B.S. 100% time

6. PROFESSIONAL INTERACTIONS:

a. Papers presented at professional meetings

Hurst, T. W. and Vulliet, P. R., Effects of Immobilization on Ventricular Fibrillation Threshold (VFT). Presented at the Joint Scientific Sessions Councils on Circulation and Basic Science, American Heart Association, Keystone, CO, February, 1986.

Vulliet, P. R., Loskutoff, N. and Kraemer, D. A Technique of Embryo Transfer in the Laboratory Rat. Annual meeting of the Western Pharmacology Society, Banff, Alberta, February, 1986.

Mitchell, J. and Vulliet, P. Phorbol Ester Activates Tyrosine Hydroxylase in the Perfused Rat Adrenal Gland. Presented at the Annual Meeting of the Society for Neuroscience Meeting, Dallas, October, 1985.

Snell, S., Kwan, S., Abell, C., Denney, R. and Vulliet, P. Monoclonal Antibodies Directed Against Tyrosine Hydroxylase. Presented at the Annual Meeting of the Society for Neuroscience, Dallas, TX, October, 1985.

Vulliet, P.R.: Phosphorylation Sites on Tyrosine Hydroxylase. Presented at the meeting of the International Congress of Biochemistry, Amsterdam, August 1985.

Vulliet, P.R.: Bioreactivity of the Adrenal Medulla. Presented at the annual meeting of the Western Pharmacological Society, January 1985.

Vulliet, P.R., and Buchl, S.J.: Biochemical regulation of norepinephrine synthesis in the myocardium. Presented at the meeting of the Basic Sciences Council, American Heart Association, Snowmass, Co., August 1984.

Hurst., T.W. and Vulliet, P.R.: Uptake of circulating epinephrine in rat myocardium. Presented at the meeting of Basic Sciences Council, American Heart Association, Snowmass, Co., August 1984.

Vulliet, P.R., Woodgett, J.R. and Cohen, P.: Tyrosine hydroxylase: phosphorylation by calmodulin-dependent multi-protein kinase (Presented at Annual Meeting of Soc. for Neurosci., Anaheim, October 1984).

b. Interactions with other laboratories

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Dept. of Human Biochemistry and Genetics
University of Texas Medical Branch
Galveston, TX 77550

7. ACQUISITION OF MAJOR EQUIPMENT:

During this time period budgeted HPLC equipment was purchased, installed and is being used for the analysis of the phosphorylated peptides and for measurement of catecholamines released from the perfused adrenal gland. A preparative centrifuge and dissection microscope have been installed. Tissue culture equipment for maintenance of pheochromocytoma and hybridoma cells has been installed.

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